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(54) Title: ASTAXANTHIN-PRODUCING YEAST CELLS, METHODS FOR THEIR PREPARATION AND THEIR USE

(57) Abstract

A yeast cell, preferably a mutant of *Phaffia rhodozyma*, which, when grown under conditions comprising an oxygen transfer rate of at least 30 mmoles/1/hour on Difco YM medium at 20-22°C for 5 days in 500 ml shake flasks with two baffles containing 50 ml of the medium and subjected to orbital shaking at 150 rpm, the inoculum being 100 µl of a four days old YM culture, produces the red pigment, astaxanthin, in an amount of at least 300 µg per g of yeast dry matter, typically at least 800 µg per g of yeast dry matter, determined by HPLC analysis using pure astaxanthin as a standard on a methanol extract of the yeast prepared by subjecting a suspension of 0.2 g of yeast dry matter in 20 ml of methanol to 5 x 1 minutes of disintegration at intervals of half a minute, the disintegration being performed at a temperature of at the most 20°C in a glass ball mill containing 15 g of glass balls having a diameter of 0.4 mm, the glass ball mill being provided with a cooling jacket with ice water. The amount of astaxanthin produced by the yeast cell, i.e. at least 300 and typically at least 800 µg per g of yeast dry matter, should be compared with the amounts of astaxanthin produced by known *Phaffia rhodozyma* strains which is at the most about 250 µg per g of yeast dry matter. The mutants of Si(Phaffia rhodozyma) may be obtained by conventional methods of mutation. The yeast cells are preferably cultivated by fed-batch fermentation which is performed under sufficient aeration, the fed-batch fermentation preferably comprising a growth phase and a subsequent growth-limited phase. Subsequent to the cultivation, the yeast cells may be subjected to e.g. rupturing and homogenization treatments, and the astaxanthin may optionally be extracted from the yeast cells. The yeast cells or the extracted astaxanthin may be used in animal feed, especially feed for anadromous fish such as salmon or trout, which when being fed with the astaxanthin-containing feed obtain an attractive red colour.

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# Astaxanthin-producing yeast cells, methods for their preparation and their use.

The present invention relates to astaxanthin-producing yeast strains, methods for their preparation, methods for their cultivation, and methods for isolating the astaxanthin from the yeast cells. Further, the invention relates to a food or feed which contains the astaxanthin-containing yeast cells or astaxanthin recovered from these as well as a method for producing food or feed and a method for feeding animals with said feed.

It is known that the red colour of the meat of anadromous fish such as salmon or sea trout is due to red pigments such as astaxanthin which is present in the feed consumed by the fish. In natural surroundings, the fish obtain their red colour from crustaceans or other astaxanthin-containing organisms, but when being bred in fish farms, the fish do not have access to these natural pigmentation sources and therefore do not obtain the attractive red colour unless red pigments are supplied in the feed.

Thus, astaxanthin isolated from crustacean wastes or produced synthetically as well as other synthetic red pigments such as cantaxanthin have been used as constituents in fish feed. However, the use of cantaxanthin in animal feedstuffs is prohibited in certain countries, and the synthetic astaxanthin production as well as the process for isolating natural astaxanthin are rather expensive and often also subject to seasonal variations.

Other natural astaxanthin sources are known, among these the yeast *Phaffia rhodozyma* and some microalgae such as the unicellular group of green algae *Chlamydomonas nivalis* [Gert Knutson et al., "Pigmentering af laks med astaxanthin fra mikroalger", Norsk Fiskeopdræt nr. 3, pp. 4-6, *55* (1980)]. The astaxanthin produced by these organisms has been shown to confer the desired red colour to anadromous fish [Eric A. Johnson et al., "*Phaffia rhodozyma* as an astaxanthin source in salmonid diets", Aquaculture, *20*, pp. 123-134 (1980) and JP-A 57-206342]. However, the use of yeast cells in large amounts as nutrition for the fish is not desirable as this feed is

not sufficiently varied. On the other hand, the amount of astaxanthin produced by the organisms and present in a nutritionally acceptable amount of yeast cells is not sufficient to obtain the desired pigmentation, and the isolation of astaxanthin from yeast by the known methods is rather expensive.

If, however, it would be possible to obtain a higher astaxanthin production from these organisms, a profitable astaxanthin production which is not subject to seasonal conditions would be possible.

The present invention provides yeast cells which contain astaxanthin in sufficiently high concentrations to make it possible to use the yeast cells as or in feed for anadromous fish and other animals in which a pigmentation of the animal meat or a product of the animal is desired. The invention also provides attractive methods for obtaining astaxanthin from astaxanthin-containing yeast cells, in particular the above-mentioned yeast cells having high contents of astaxanthin. Important aspects of the invention are based on a particular method for cultivating astaxanthin-producing cells and/or the provision of mutant strains with an improved inherent capability of producing astaxanthin.

Thus, one aspect of the invention relates to a yeast cell which, when grown under conditions comprising an oxygen transfer rate of at least 30 mmoles/l/hour on Difco YM medium at 20-22°C for 5 days in 500 ml shake flasks with two baffles containing 50 ml of the medium and subjected to orbital shaking at 150 rpm, the inoculum being 100 µl of a four days old culture in YM-medium, produces astaxanthin in an amount of at least 300 µg per g of yeast dry matter, determined by HPLC analysis using pure astaxanthin as a standard on a methanol extract of the yeast prepared by subjecting a suspension of 0.2 g of yeast dry matter in 20 ml of methanol to 5 x 1 minutes of disintegration at intervals of half a minute, the disintegration being performed at a temperature of at the most 20°C in a glass ball mill containing 15 g of glass balls having a diameter of 0.4 mm, the glass ball mill being provided with a cooling jacket with ice water.

The growth conditions and determination conditions stated above are given to standardize the growth and testing methods so that the result obtained will reflect the inherent astaxanthin-producing capabilities of the yeast in question. This method has been found, by several experiments performed by the applicant company, to be a suitable and reproducible method which is easy to perform in practice. It should be noted that the determination method is not the same as the one hitherto used in the literature. The methods hitherto used in the literature, cf. e.g. Eric A. Johnson et al., "Astaxanthin formation by the yeast Phaffia rhodozyma", *Journal of general microbiology 115*, 1979, pp. 173-83, are based on the absorbance of a 1% (w/v) solution in acetone in a 1 cm cuvette of 1600, whereas the value, which is obtained by measuring the astaxanthin standard from Hoffmann-La Roche, is 2100. This value is based on the applicant company's own measurements as well as on the information given by Hoffmann-La Roche.

Furthermore, the known determination method measures the total pigment content of the yeast whereas the above-mentioned standard method used in the present application exclusively measures the astaxanthin content. When comparing the values obtained by the standardized method as stated above with the values stated in the literature, it should be borne in mind that the values stated in the literature will be considerably higher than the true values obtained by the standardized method stated above. Thus, whenever the present total pigment content is compared with the literature statements, a correction for the difference in extinction coefficients should be made by multiplying the total pigment content stated in the literature by 1600/2100.

The growth conditions stated above are the ones which have been found by the applicant company to be reproducible and significant for the determination of the inherent astaxanthin-producing capability. A more detailed explanation of the growth and determination conditions used for determining the inherent astaxanthin-producing capabilities of yeast strains is given in connection with the Examples.

The yeast cell according to the invention is preferably a yeast cell which belongs to the genus *Phaffia* and in particular one which belongs to the species *Phaffia rhodozyma* as this is the only *Phaffia* species known for the time being.

At present, *Phaffia rhodozyma* is the only known yeast which produces astaxanthin. The wild-type *P. rhodozyma* is isolated from deciduous tree exudates and an example of such a wild-type strain is deposited in the American Type Culture Collection under the accession number ATCC 24261.

Vegetative *P. rhodozyma* cells form buds as heterobasidiomycetous yeast. Clamydospores are developed by budding but promycelium and proper spore formation do not occur. The chlamydospores are relatively large spherical cells with a larger lipid content than the vegetative cells. Attempts to pair the various strains in the hope of observing dikaryotic mycelium and teliospore formation have not been successful. *P. rhodozyma* was therefore classified in the genus *Deuteromycotina* of the order *Blastomycetes* (cf. M.W. Miller et al., "Phaffia, a new yeast genus in the deuteromycotina (Blastomycetes)", in *International Journal of systematic bacteriology* 26:2, 1976, pp. 286-291).

Vegetative cells are ellipsoidal (3.6-7.5) x (5.5-10.5) µm and are present in a liquid medium individually, in pairs and in some cases in short chains or small clusters. No true mycelium is developed, but a rudimentary pseudomycelium may be present. Budding occurs several times from the same point on the cell. *P. rhodozyma* has a strong cell membrane composed by many layers, and capsule material imparts a granular appearance to the surface and causes the clustering mentioned above.

A sexual cycle of life has not been observed. During the development of the chlamydospores, vegetative cells are formed by budding. These cells cannot be considered to be promycelia with spores as described for *Aessosporon* (cf. J.P. van der Walt, "The perfect and imperfect states of *Sporobolomyces salminicolor*", *J. Microbiol. Serol. 36*, 1970, pp. 49-55). The chlamydospores cannot be considered to be gono-

toconter (sexually segregated spores), and their buds cannot be considered to be the haploid generation. It has not been possible by nuclear staining to demonstrate diploidization at any growth stage. Transmission electron micrographs have only shown one single nucleus during all growth phases (cf. M.W. Miller et al., *op.cit.*). Thus, *P. rhodozyma* is likely to be haploid, but this has not been proved.

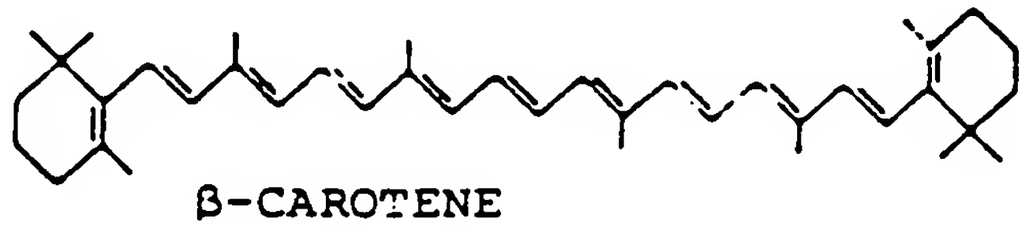
After 2-4 weeks of growth on YM agar (Difco Laboratories Incorporated, Difco manual: dehydrated culture media and reagents for microbiology, 10th Edition, Detroit 1984), the string cultures are orange to salmon-pink, depending on the strain.

*P. rhodozyma* has the special property of not growing at temperatures above 27°C. It ferments D-glucose, maltose, sucrose and raffinose whereas D-galactose and melibiose are not fermented. The most common carbon sources are assimilated; however, D-galactose, L-sorbose, melibiose, lactose, glycerol and citrate are not assimilated. The yeast cannot grow in vitamin-free medium without the addition of biotin (M.W. Miller et al., *op.cit.*). The most common nitrogen sources are assimilated, including urea. Potassium nitrate and ethylamine are not assimilated. The yeast cannot grow on 50% by weight of a glucose-yeast extract agar nor on 10% by weight of sodium chloride-yeast extract agar. The acid formation on chalk agar by the yeast is weak and so is the gelatin liquefaction. Casein hydrolysis, depolytic activity and growth in the presence of 0.1 µg of cycloheximid per ml are absent whereas the yeast is able to synthesize starch-like compounds independent of pH. The mole-% G+C is measured to be 48.3 ± 0.18 (cf. Miller et al., *op. cit.*).

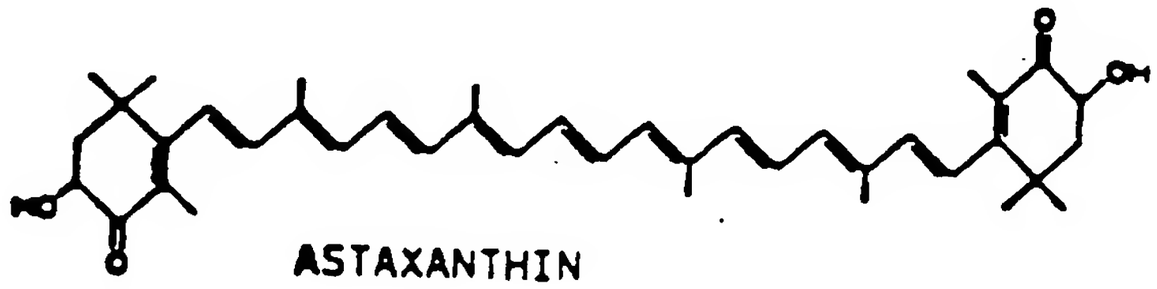
During growth under carbohydrate- and/or nitrogen-limited conditions, when subjected to fed-batch fermentations, *P. rhodozyma* produces trehalose as a carbohydrate deposit. This is quite a new observation made by the applicant company and has not been reported hitherto.

*P. rhodozyma* produces a number of carotenoids, of which astaxanthin constitutes 83-87%, β-carotene 2-2.5%, echinenone 2-4% and phoenicoxanthin 5-7%, according to the literature. In practice, the ratio of astaxanthin to total pigment produced by *P. rhodozyma* has, however,

been found to vary considerably depending on the growth conditions of the yeast cells as well as the pigment determination method and has generally been found to be in the range of 50-80%.



β-CAROTENE



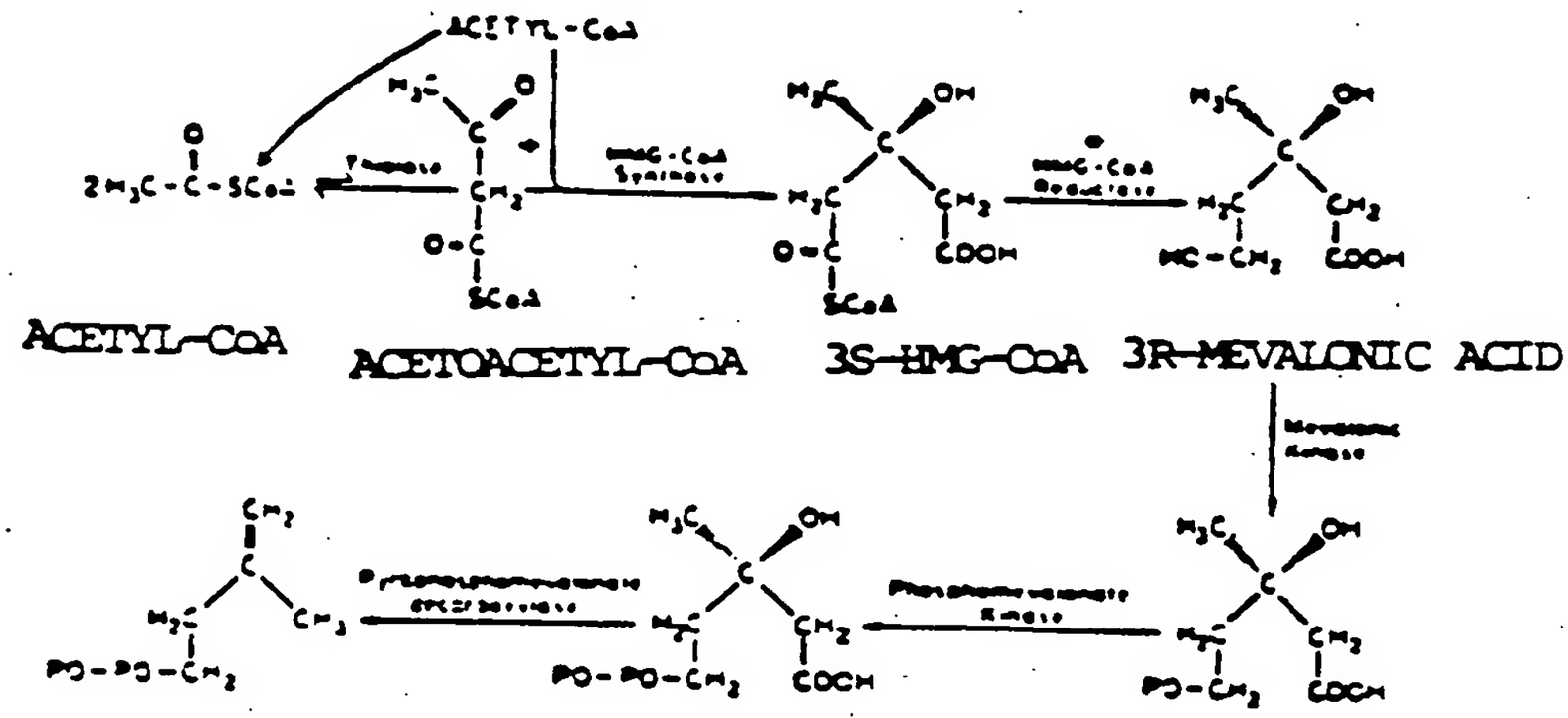
ASTAXANTHIN

All hydroxylated pigments, including astaxanthin, have been described as non-bound, not as esters or other derivatives (Arthur G. Andrewes et al., "Carotenoids of Phaffia rhodozyma, a red-pigmented fermenting yeast", in *Phytochemistry 15*, 1976, pp. 1003-1007). There exist three optical isomeric forms of astaxanthin: (3S,3'S), (3R,3'R) and (3S,3R), each existing in various trans- and cis-configurations. It has been reported that *P. rhodozyma* only produces (3R,3'R)-astaxanthin (Arthur G. Andrewes et al., "(3R,3'R)-astaxanthin from the yeast Phaffia rhodozyma", *op. cit.*, pp. 1009-1011). In the present context, "astaxanthin" is used about trans- as well as cis-configurations of astaxanthin.

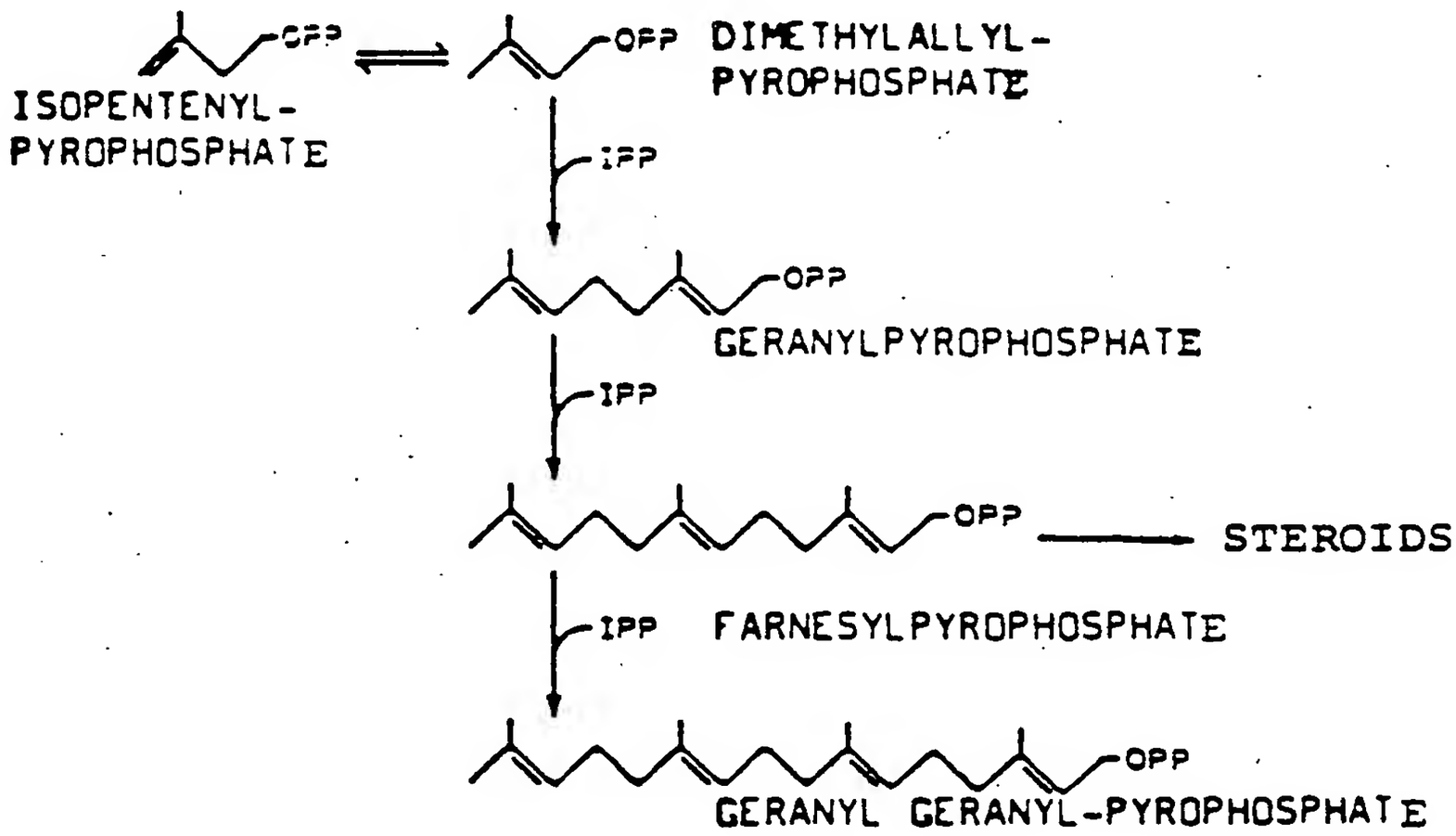
The pigment in the individual *P. rhodozyma* cells is not visible when the cells are studied in a microscope, which indicates that the pigment may be dispersed throughout the cell. However, it is also possible that the pigment is concentrated in certain parts of the cells.

Astaxanthin is an oxidated carotenoid and therefore belongs to the xanthophyl group. Similarly to other carotenoids, astaxanthin is composed of eight isoprenoid units. By the biosynthesis of astaxanthin

which is catabolite repressed, isopentenyl pyrophosphate is formed from acetyl-CoA as illustrated below.

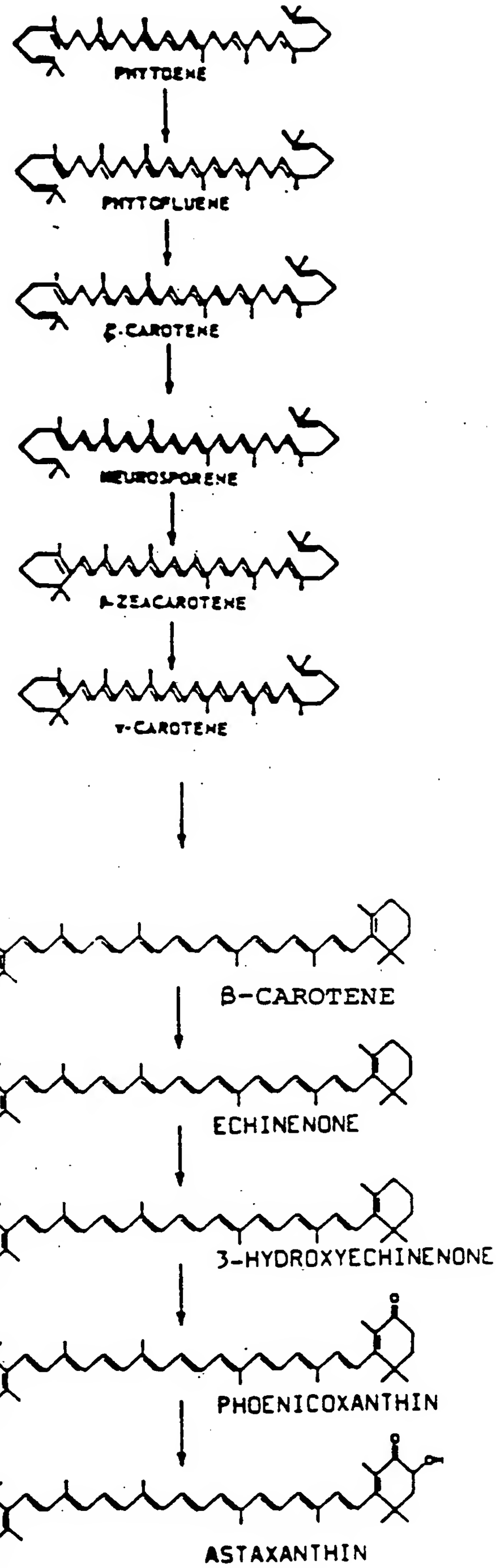



By three prenyltransferase reactions, isopentenyl pyrophosphate forms geranyl geranyl pyrophosphate via geranyl pyrophosphate and farnesyl pyrophosphate as illustrated below.




Condensation of two-molecular geranyl geranyl pyrophosphate forms phytoene which, via dehydrogenation steps and ring forming, forms astaxanthin from $\beta$-carotene. The last part of the biosynthesis has not been unambiguously determined, but Andrewes et al. (*op.cit.*) have proposed the metabolism route shown below on the basis of the pigment

composition in *P. rhodozyma*.


PHYTOENE
PHYTOFLUENE
ζ-CAROTENE
NEUROSPORENE
δ-ZEACAROTENE
γ-CAROTENE
β-CAROTENE
ECHINENONE
3-HYDROXYECHINENONE
PHOENICOXANTHIN
ASTAXANTHIN

The enzyme system which converts geranyl geranyl pyrophosphate to astaxanthin is not known, and it is therefore not known why *P. rhodozyma* produces (3R,3'R)-astaxanthin and whether there are some regulating steps during this part of the biosynthesis. On the other hand, the conversion of acetyl-CoA to isopentenyl pyrophosphate in other isoprenoid-producing organisms than *P. rhodozyma* and the enzymes which take part have been described in relatively great detail. Less is known about the enzymes which convert isopentenyl pyrophosphate to geranyl geranyl pyrophosphate (isopentenyl pyrophosphate isomerase and prenyltransferase (J.W. Porter, S.L. Spurgeon (eds.), "Biosynthesis of isoprenoid compounds". New York, 1981-1983).

The protein content of *P. rhodozyma* varies from 25 to 50% of yeast dry matter, depending on the culturing conditions. This is a relatively low protein content. In contrast to this, the lipid content is extraordinarily high (14-27%). It is contemplated that the nucleic acid constitutes 8% similarly to other yeasts and that the amino acid composition is similar to the composition in other known yeasts such as *Saccharomyces cerevisiae* and thus has a very low content of certain amino acids, e.g. methionin and cystein (cf. Gerald Reed and Henry J. Peppler, *Yeast Technology*, 1973, p. 329, published by The AVI Publishing Company, Inc.). This and the overall yeast composition which comprises a high amount of nucleic acids make the yeast inconvenient for animal nutrition purposes when the yeast is the only nutrient source, such as indicated above. Thus, without addition of certain amino acids and other nutrient components, the yeast will not be a suitable major nutrition component for fish or other animals.

The total amount of astaxanthin which is produced by the wild type *P. rhodozyma* when this is grown under the normal known conditions is sufficient to confer a red colour to the yeast cell but is not sufficient to make recovery of the astaxanthin from the yeast cells economically feasible.

None of the *Phaffia rhodozyma* species described in the literature have an inherent astaxanthin-producing capability of more than 300 µg per g of yeast dry matter when analyzed in accordance with the above standard methods, *vide* Table 2 and Table 6 of the examples. However, according to the present invention, it has been found possible to obtain yeast cells which are inherently capable of producing astaxanthin in an amount of at least 450 µg per g of yeast dry matter, such as at least 600 µg per g of yeast dry matter, preferably at least 700 µg per g of yeast dry matter, more preferably at least 1000 µg per g of yeast dry matter, especially at least 1500 µg per g of yeast dry matter, and most preferably at least 2000 µg per g of yeast dry matter, the growth and the determination being performed by the standard methods stated above. These yeast cells have been produced from naturally occurring *Phaffia rhodozyma* by mutagenization. Thus, an aspect of the present invention relates to a method for producing a yeast cell showing the high inherent astaxanthin-producing capability explained above, the method comprising treating a yeast cell with a mutagen and selecting a resulting mutant which, when grown under the conditions stated above, is capable of producing astaxanthin in an amount of at least 300 µg per g of yeast dry matter, determined by the method stated above.

The mutagenization may be performed as a single mutagenization, but it has been found advantageous to perform two or more consecutive mutagenizations, as it has been found that the inherent capability of producing astaxanthin may be improved by each mutagenization step.

The starting yeast cell subjected to mutagenization is normally a yeast cell which, when grown under the conditions stated above, produces astaxanthin in an amount of less than 300 µg per g of yeast dry matter, determined by the method stated above, but it is evident that a normal candidate for the mutagenization treatment will be a naturally occurring yeast cell having as high inherent astaxanthin production as possible. Such yeast cells are normally yeast cells which belong to the genus *Phaffia*, in particular yeast cells belonging to the species *Phaffia rhodozyma*, such as is mentioned above.

The mutagenization treatment may be performed using any suitable mutagen (in the present context, the term "mutagen" is to be understood

in its broad sense as comprising, e.g., not only agents which have a mutagen effect, but also treatment which have a mutagen effect such as UV irradiation). Examples of suitable mutagens are ethyl methane sulphonate, UV irradiation, N-methyl-N'-nitro-N-nitrosoguanidine, nucleotide base analogues such as bromouracil, and acridines, but it is contemplated that any other effective mutagen will be suitable for the treatment.

In accordance with conventional mutagenization techniques, the mutagenization is followed by suitable selection of the cells which have the highest astaxanthin production. Due to the fact that astaxanthin is a pigment, this selection may be performed relatively easily by normal visual means, such as simple observation of single colonies. An alternative method is to perform analysis on cultures made from single colonies, e.g. by using the standardized cultivation conditions and determination conditions as explained above.

Two strains produced by the mutagenization method according to the invention and showing a particularly high astaxanthin productivity were deposited on 6 April, 1987 at the Centraalbureau voor Schimmelcultures, Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands (CBS) under the accession Nos. 224-87 and 225-87, respectively, and one strain being a reisolate of CBS 225-87 (*vide* Example 1) was deposited on 23 March, 1988 at CBS under the accession No. 215-88, and an aspect of the invention relates to these yeast strains as well as mutants or derivatives thereof which have substantially retained or improved the astaxanthin-producing capability of these strains.

The invention also relates to a method for producing astaxanthin-containing yeast cells or cell parts, or astaxanthin derived from these yeast cell or cell parts. This method comprises cultivating astaxanthin-producing yeast cells under aerobic conditions in a medium containing carbohydrate sources, assimilable sources of nitrogen and phosphorus, micronutrients and biotin or desthiobiotin at a temperature in the range of 15-26°C so as to obtain a biomass containing astaxanthin in an amount of at least 300 µg per g of yeast dry matter, determined by the method stated above,

and optionally performing one or several of the following steps in arbitrary sequence:

- harvesting cells from the culture so as to obtain a yeast cream,
- opening the cells, for example rupturing the cell walls by means of mechanical, chemical and/or enzymatic treatment and/or subjecting the cells to sonication, autolysis, osmolysis and/or plasmolysis optionally with addition of suitable agents such as detergents, acids, bases, enzymes, autolysis-enhancing substances, osmolysing agents such as salts, and/or plasmolysing agents,
- homogenizing the cells to obtain a homogenate,
- drying the cells, the cell fragments or the homogenate, preferably to a water content of at the most 12% by weight, preferably at the most 10% by weight,
- extracting astaxanthin from the cells, the cell fragments or the homogenate.

The amount of astaxanthin stated above, 300 µg per g of yeast dry matter, is higher than any astaxanthin concentration reported in the literature. Although Johnson et al., *op. cit.*, reports an astaxanthin content of 295 µg per g of yeast dry matter, this value does not only comprise the astaxanthin content but in fact the total pigment content of the yeast cell. Further, this pigment content was measured using a value of the absorbance of a 1% (w/v) solution in acetone in 1 cm cuvette of 1600 which is lower than the one measured by the present applicants (2100) so that the value reported by Johnson et al. corresponds to at the very most 295 x 1600/2100 = 225 µg of total pigment (not only astaxanthin) per g of yeast dry matter. This pigment content from the literature should be compared with the total pigment content of the yeast strains of the present invention which is 885 µg/g of yeast dry matter for the strain CBS 224-87, 1176 µg/g of yeast dry matter for the strain CBS 225-87, and about 1340 µg/g of yeast dry matter for the strain CBS 215-88, or even higher, e.g. at least 2000 µg/g of yeast dry matter.

The high astaxanthin concentration in the yeast cells of the invention may be obtained partly by the use of special cultivation

conditions as explained below and partly by selecting a yeast strain with a high inherent astaxanthin productivity, preferably a yeast strain as discussed above, and in particular it is preferred to combine the special cultivation conditions and the use of special astaxanthin-producing yeast strains.

The cultivation is preferably performed as a fed-batch fermentation under conditions where substantially no alcohol is formed. As mentioned above, the temperature of the culture is in the range of 15-26°C. Below 15°C, the growth tends to be too slow to be acceptable for industrial production, and above 26°C, the viability of the culture is severely impaired. The preferred temperature range is 20-22°C.

The fermentation or at least part thereof is normally performed in a medium which comprises suitable macro- and micronutrients for the cells, such as molasses or saccharose as a carbohydrate source and nitrogen sources such as corn-steep-liquor, diammonium sulphate, ammonium phosphate, ammonium hydroxide or urea, phosphorus sources such as ammonium phosphate and phosphoric acid and added micronutrients or mineral salts such as magnesium sulphate, zinc sulphate and biotin or desthiobiotin. The molasses or saccharose are preferably supplied to the medium separately from the other components in accordance with conventional methods used in yeast production. When the medium comprises molasses, it has been found that the growth of the yeast cells is affected by the concentration of sugar or other growth-inhibiting substances therein in the fermenter. This effect has not been observed when the medium comprises corn-steep-liquor or solids. Accordingly, it may prove advantageous to regulate the fermentation so that the concentration of sugar (expressed as the total concentration of glucose and saccharose) in the fermenter is at the most 8 g/l, preferably at the most 5 g/l, and most preferably at the most 1 g/l.

The culture is aerated during the total fermentation, i.e. it is grown under aerobic conditions. By the term "aerobic conditions" is meant that the oxygen supply should be sufficient so that substantially no oxygen limitation will occur during the fermentation.

According to a special aspect of the invention as indicated above, the concentration of astaxanthin in the biomass obtained is increased by performing the cultivation under selected conditions. These conditions involve a cultivation which comprises a growth phase under conditions which are substantially sufficient with respect to substantially all growth conditions and a subsequent growth-limited phase. The growth-limited phase is preferably established by providing conditions where the growth medium under continued aeration is deprived of at least one growth factor so as to enhance the production of astaxanthin during the subsequent phase.

The growth-limited phase should be understood to generally mean the phase in which the main part of the cells have stopped growing. This phase does of course occur when the medium is deprived of at least one growth factor but is also observed during the last part of the period of carbohydrate addition when the amount of cells present in the fermenter is well in excess of the aeration capacity of the fermenter.

It is not known why the subsequent growth-limited phase has the surprising effect of considerably enhancing the production of astaxanthin (for example from 231 to 369 µg per g of yeast dry matter as obtained in one of the examples which follows), but it is contemplated that the precursors of astaxanthin have been produced during the growth phase, and that the subsequent growth-limited phase provides conditions which promote the final production of astaxanthin, possibly oxidizing conditions with the surplus of oxygen which becomes available when the growth is terminated. At any rate, it seems essential that aeration is continued during the subsequent growth-limited phase. The duration of the subsequent growth-limited phase is preferably at least about 16 hours, such as 16-24 hours, as shorter durations may tend to decrease the extra effect obtainable, whereas there seems to be no substantial effect obtainable by extending the growth-limited phase to more than about 24 hours.

Expressed in a functional manner, the conditions of the growth-limited phase should be adapted to enhance the astaxanthin production to

at least 1.2 times the production obtained without the subsequent phase, such as at least 1.3 times the production obtained without the subsequent phase, preferably at least 1.4 times the production obtained without the subsequent phase and most preferably at least 1.5 times the production obtained without the subsequent phase.

While the yeast cell subjected to the special cultivation with the subsequent growth-limited phase may be a wild-type astaxanthin-producing yeast cell whose astaxanthin productivity is increased due to the subsequent growth-limiting step, such as a wild-type yeast cell of the genus *Phaffia*, in particular of the species *Phaffia rhodozyma*, it is preferred that the yeast cell subjected to the cultivation is a yeast cell having an inherent and improved capability of producing astaxanthin, typically a yeast cell obtained by mutagenization as explained above. With these yeast cells with an inherent increased astaxanthin production, the concentration of astaxanthin in the biomass obtained when using the special cultivation method comprising a growth-limited phase may be at least 600, preferably at least 800, more preferably at least 1000 µg per g of yeast dry matter, especially at least 1500 µg per g of yeast dry matter, e.g. at least 2000 µg per g of yeast dry matter, and most preferably at least 3000 µg per g of yeast dry matter, determined as stated above.

Normally, and as used above, the total pigment content and astaxanthin content of the yeast cells or yeast cell parts are stated as µg/g of yeast dry matter. However, other ways of stating the total pigment and astaxanthin content may be found convenient. It may, e.g., be useful to state the total pigment content and astaxanthin content as µg/ml of the suspension in which it is present, e.g. in the growth media. Thereby, it will not be necessary to determine the weight of yeast dry matter of the yeast cells from which the astaxanthin or total pigment is recovered. Thus, during the fermentation or cultivation of the yeast cells, the astaxanthin and/or total pigment content of the yeast cells may easily be determined.

After the cultivation as described above to obtain yeast cells having a high astaxanthin content, the culture may be subjected to the sub-

sequent treatments mentioned above to isolate the yeast cells and/or condition them for their subsequent use, such as by rupturing the cells, and/or astaxanthin may be extracted from the cells.

The following important examples of these treatments are discussed in greater detail:

The cells may be ruptured by subjecting the cells to an increased pressure and then releasing the pressure.

The cells may be subjected to the increased pressure and release of the pressure by passage through a system comprising a valve homogenizer where the increased pressure is built up in front of the valve homogenizer. The valve homogenizer typically comprises an aerojet through which the cell suspension is passed under high pressure and an obstruction member which the jet hits substantially after passage through the valve. Examples of cell disruption valves are described in APV Gaulin Technical Bulletin No. 74 of March 1985 (APV Gaulin International SA, P.O. Box 58, 1200 AB Hilversum, the Netherlands), incorporated by reference herein. As an example of a suitable cell rupture homogenizer may be mentioned an APV Gaulin MC4 homogenizer with a cell rupture valve of the type CR as described in the above-mentioned publication. The homogenizer is connected to a a heat exchanger in which the suspension comprising ruptured cells passes from the cell rupture valve. The pressure of the cell suspension in front of the valve may, e.g., be about 400-1200 bar, such as, e.g., about 700 bar. This treatment may for example be repeated three times with intervening cooling of the homogenate in the heat exchanger.

As is explained below, it is necessary that the cells are ruptured or otherwise treated when they are to be used in feed as the utilization of the astaxanthin content to a high degree depends on the cell contents being available to the digestive system of the animal in question. Thus, substantially no pigmenting effect is obtained when feeding fish with feed containing non-ruptured astaxanthin-containing cells.

The ruptured yeast cells may be subjected to ultrafiltration or evaporation so as to concentrate the ruptured cells. The ultrafiltration may, e.g., be performed in a lab unit system available from De Danske Sukkerfabrikker, for example a System 37 which comprises three filtering units of a total filter area of 0.88 $m^2$ of an ultrafiltration membrane of the type RC 70. Another method for concentrating the ruptured cells is to perform vacuum evaporation of water from the cell suspension.

The ruptured cells may be dried by spray drying or drum drying. Before drying, carriers such as sodium caseinate, antioxidants and/or emulsifiers are preferably added. Spray drying may, e.g., be performed by subjecting a homogeneously mixed slurry of the ruptured cells and optionally a carrier such as sodium caseinate, preferably in the form of an aqueous solution, to spray drying. The spray drying may suitably be carried out by mixing the aqueous sodium caseinate solution with the yeast slurry so as to obtain a sodium caseinate concentration of about 2-10% (w/v). The resulting mixture is then allowed to stand with stirring in a nitrogen atmosphere before being pumped into a spray drying tower in which it is subjected to drying at a temperature of, e.g., 150-230°C, such as about 180°C to decrease the water content of the yeast cell material to e.g. at the most 10% by weight. The yeast cell material is then subsequently atomized by means of a spray wheel. The powdery yeast material resulting from the spray drying treatment is suitably recovered by means of cyclone and optionally subsequently sieved and packed. An example of a suitable spray drying equipment is a spray tower of the type EAK-1 from Anhydro. As an alternative, the ruptured yeast cells may be subjected to drum drying, for example in a closed drum drying equipment at a temperature of 150-200°C.

As the astaxanthin is very easily decomposed at high temperatures, it is important that the ruptured yeast cells are subjected to high temperatures for as short a time as possible. Further, as astaxanthin is sensitive to oxygen, the drying should preferably be performed under non-oxidizing conditions, for example in an inert atmosphere such as water vapour (which may be the water vapour evaporated from the yeast suspension), nitrogen, and/or carbon dioxide.

Prior to drying, the ruptured cells are optionally mixed with suitable emulsifiers such as sorbitan monostearate or antioxidants, butyl hydroxytoluene (BHT), butyl hydroxyanisol (BHA), vitamin E, ascorbic acid, (II) sulphate or (II) phosphate esters of ascorbic acid, or ascorbyl palmitate.

Dried ruptured cells are immediately useful as a constituent of animal feed, such as is explained below.

The astaxanthin content of the yeast cell may be extracted from these by use of various extraction agents and extraction procedures - so as to ensure that a substantial total extraction of the astaxanthin from the yeast cells is obtained. In most cases, the extraction has to be performed in ruptured cell material. Thus, the ruptured cell material, which may be dry or wet, may be extracted with an organic solvent such as petroleum ether which is suitably employed in the case of wet cell material as the petroleum ether forms a phase separately from the water phase. Other suitable organic solvents are acetone or alcohols such as methanol or ethanol, ethers, ketones and chlorinated hydrocarbons. By the extraction, astaxanthin is dissolved in the organic solvent. The astaxanthin may be obtained by removing the solvent from the solution such as by evaporation in a falling film evaporation system before drying. However, also a concentrate of astaxanthin in the organic solvent may be convenient for certain purposes. A concentrate may be used *per se* in the production of feed or food, or the concentrate may be diluted and used in the diluted state in the preparation of feed or food, for example by impregnating feed or food constituents with the solution or by using the solution (or the concentrate) for colouring food constituents such as oils or fats.

The astaxanthin may also be extracted from yeast cells by use of carbondioxides under supercritical conditions. The carbondioxide may optionally be used in combination with suitable entrainers such as organic solvents, especially solvents of the above mentioned types, or solvents such as chloroform or acetonitrile, or glacial acetic acid. The yeast cells subjected to supercritical extraction may be

wet or dry whole yeast cells or ruptured, e.g. homogenized, yeast cells.

A preferred method of isolating whole astaxanthin-containing cells from the culture is to filtrate the yeast cream, for example on a filter press or a rotating drum filter, so as to obtain a filter cake, e.g. with a dry matter content of about 25-35%. The filter cake may then suitably be extruded into strings, for example strings with a diameter of about 0.5-2.0 mm in an extruder equipped with a perforated plate, so as to obtain strings consisting of yeast particles. The strings are preferably extruded directly into the hot air in a fluid bed where they are dried. The evaporation in the fluid bed is preferably regulated so that the temperature of the yeast particles is kept below 50°C such as at 30-40°C, and the process is terminated when the water content is brought down below 10% by weight, preferably below 8%, as determined by the yeast dry matter content (the procedure is described in the Examples). Alternatively, the drying may be performed in a tray drier under the same conditions as in the fluid bed. The dried whole cell material may then be comminuted in a ball stirring mill such as a Coball® mill after which it is subjected to extraction.

According to a special method, whole cell dried material, for example obtained as described above, may be mixed with an oily phase such as an edible oil or fat such as soy bean oil or fish oil, or another organic solvent such as a solvent of the type discussed above. The temperature is preferably in the range of 20-30°C. The mixture obtained from the cell material and the oily phase or the organic solvent may be ground in a mill such as a ball mill, e.g. a ball stirring mill such as a Coball® mill, to rupture the cells and release astaxanthin from the cells. The resulting suspension may be used as such in feed, or the oily phase containing the astaxanthin may be separated from cell residues before use. The separation is suitably performed by centrifugation in a fast running centrifuge, the same principle which is employed in separation of bacteria from wort. Another possibility is of course to mix ruptured dried cell material obtained by the methods discussed above with an oily phase in a similar manner to extract the astaxanthin into the oily phase

and perform separation as described above. The oily phase may be used for colouring feed in the same manner as described above.

In contrast to most conventional extraction procedures which, as stated above, has to be performed on ruptured cell material, it has been found that glacial acetic acid successfully may be employed to extract astaxanthin from whole, non-ruptured yeast cells. Thus, according to one aspect of the present invention, astaxanthin may be extracted from whole yeast cells with a solvent comprising glacial acetic acid, the extraction preferably being performed at a temperature above the freezing point of the solvent, e.g. in the range of 20-100°C, preferably in the range of 20-80°C, and more preferably in the range of 20-60°C. It is contemplated that it is possible to obtain a more selective extraction of astaxanthin when the extraction is performed at the lower temperatures as concomitant extraction of fat and other extractable components will be limited at these low temperatures. The concentration of glacial acetic acid in the solvent is preferably in the range of 5-100, 10-70. The extraction with glacial acetic acid results in an extraction of the pigment of the cells of about 70-90%, i.e. substantially all the pigment and astaxanthin contents of the yeast cells are found in the glacial acetic acid extract. In addition, the extract normally contains about 30-35% of yeast dry matter. Suitably, the yeast subjected to extraction with glacial acetic acid is in the form of dried yeast, e.g. yeast which has been filtered and subsequently extruded into a fluid bed wherein it is dried, as thus treated yeast cells will not rupture during the extraction treatment (unless the extraction treatment involves vigorous mechanical treatment of the yeast cells). This will facilitate the subsequent separation of the extract containing the pigment from the yeast cells as compared with extraction of ruptured or homogenized cells, which, due to their relatively small sizes in comparison with non-ruptured cells to a large extend tend to block up the pores of the filter employed. The glacial acetic acid extraction is illustrated in Example 9.
Extraction of wet yeast cells with glacial acetic acid may also prove useful.

The extracted astaxanthin as well as the whole dried cell material are preferably kept under oxygen-deficient conditions so as to protect the astaxanthin from decomposition. Thus, the astaxanthin-containing yeast cells or the extracted astaxanthin is preferably protected by means of antioxidants such as butyl hydroxyanisol (BHA), butyl hydroxytoluene (BHT), vitamin E or ascorbic acid, (II) sulphate or (II) phosphate esters of ascorbic acid, or ascorbyl palmitate, and/or emulsifiers such as monoglycerides or sorbitan esters and are suitably kept under hermetic conditions.

The invention also relates to an animal feed comprising yeast cells or yeast cell parts containing astaxanthin in an amount of at least 300 µg per g of yeast dry matter, determined as explained above, in combination with other feed constituents. Preferably, the astaxanthin-containing yeast cells or yeast cell parts constitute at the most 10% by weight of the dry matter of the total animal feed composition, preferably at the most 5% and more preferably at the most 3%. These values are calculated on the final feed to be administered to the animals. It is also possible to prepare feed premixes having a higher concentration of yeast cells. The yeast cells or yeast cell parts or the astaxanthin is optionally admixed with emulsifiers which are capable of making the astaxanthin dispersible in water. In addition, the astaxanthin-containing yeast cells or yeast cell parts may be protected against oxidation by means of the antioxidants and/or emulsifiers mentioned above, and/or the animal feed may be packaged in air-tight and optionally evacuated containers.

The astaxanthin-containing dried yeast cells may also be packaged *per se* for use as a feed constituent, the final feed mixture being prepared at the site of use, or the yeast cells being administered *per se* to animals which are otherwise fed with normal or adapted feed mixtures.

The yeast cells or yeast cell parts are suitably and normally mixed with other nutrient components which are preferably selected from protein and carbohydrate sources, fats or oils and micronutrients such as vitamins and minerals. As examples of protein sources may be

mentioned casein, albumin, wheat gluten, fish meals, concentrated fish residues (fish glue meal and blood meal). As examples of carbohydrate sources may be mentioned gelatinized starch, extruded wheat, molasses, vegetable flours and corn starch. The fat constituents in the feed may for example be fish oil and cod liver oil and/or vegetable oils such as corn oil. The minerals may be selected, e.g., from inorganic or simple organic compounds of calcium, phosphorus, sodium, potassium, chlorine, magnesium, copper, manganese, zink, cobalt and selenium. As examples of vitamins may be mentioned vitamin $B_{12}$, proline, vitamin A, vitamin D, vitamin E, vitamin K, thiamine, ascorbic acid, riboflavine, pyridoxine, panthotenic acid, niacine, biotin, choline and inositol.

The invention also relates to food or feed comprising astaxanthin which has been extracted from yeast cells, for example by any of the methods described above, preferably from yeast cells according to the invention or yeast cells produced by the method of the invention. The astaxanthin may be used in admixture with the feed constituents described above and also in admixture with other food or nutrient components as well as in admixture with other colourants. Thus, astaxanthin extracted from yeast cells is well suited alone or in combination with other colourants for use in edible oils, butter, margarine, shortening, mayonnaise, patés, soups, snack products, surimi-based products, desserts, ice cream, confectionery, baked products, and beverages. When the astaxanthin is used in food which is mostly constituted by water or water phases, the astaxanthin is preferably mixed with an emulsifier as discussed above which makes the astaxanthin dispersible in the water phase without any tendency to crystallize and without the necessity of adding an oily phase to dissolve the astaxanthin.

Furthermore, the invention relates to a method for feeding animals to obtain a reddish pigmentation of their meat and/or of products produced by the animals, comprising administering to the animals a feed containing yeast cells or cell parts containing astaxanthin in an amount of at least 300 µg per g of yeast dry matter, determined by the method stated above, or astaxanthin extracted from such yeast cells or cell parts.

The amount of the feed containing the astaxanthin or the astaxanthin-containing yeast cells or cell parts administered to the animals will depend upon the animal species in question and upon the pigmentation effect which it is desired to obtain by means of the astaxanthin. Evidently, the principle to be followed is that the animal should have a normal recommended daily ration of macro- and micronutrients and, in addition, astaxanthin in a form and an amount which will result in the desired pigmentation of the animal meat or the animal product in question. In some cases, the amount of astaxanthin to be administered will depend on the season; thus, for example, it will normally not be preferred to administer astaxanthin or other carotenoids to cows to obtain a pigmentation of the butter in the summertime as the butter pigmentation is normally considered adequate when the cows are grazing. Also the amount in which the feed containing the astaxanthin or the astaxanthin-containing yeast cells or cell parts is administered to the animals may in some cases be dependent on the season. Thus, for example in the case of fish such as salmon or sea trout, the amount of feed consumed by the fish in the wintertime is relatively low which is in contrast to the amount consumed by the fish in the summertime. However, a suitable amount of feed administered to the fish may be about 1.5% of fish body weight per day which corresponds to the recommendations given by the California State Department of Fish and Game.

When feeding poultry by the method stated above in order to pigment the yolks of the eggs produced by the poultry and/or the meat or skin of the poultry, the feed may be constituted by conventional poultry feed components, an example of which is one which is preferably constituted by protein and carbohydrate sources such as soy bean meal, soy bean protein, cellulose, starch and fat sources such as soy bean oil, vitamins such as an overall vitamin mix and minerals such as a mixture of the common mineral components for poultry as well as calcium sources for the egg shells, the calcium sources preferably being calcium carbonate and calcium hydrogen phosphate. A small amount of sodium chloride may also be present. The feed may be administered in a conventional dosage.